BIOCHEMICAL BASIS OF RESISTANCE IN SMALL GRAINS TO THE GREENBUG APHID, SCHIZAPHIS GRAMINUM

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Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1975

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ACKNOWLEDGEMENTS

The author gratefully acknowledges the guidance and stimulating advice of his major professor, Dr. Robert K. Gholson during the course of this work. Sincere thanks are extended to Dr. George V. Odell and Dr. Roger E. Koeppe whose help and wonderful advice during my stay in the Biochemistry Department made it a worthwhile experience.

Appreciation is also expressed to Dr. K. J. Starks and Mr. R. L. Burton for their fine cooperation on this project. Special thanks are due to Dr. B. G. Hudson for his help in the use of the Carbohydrate Analyzer and to Dr. D. Colton for carrying out the quantitative periodate oxidations. The excellent assistance of Mr. Keith Kinnerberg in obtaining the mass spectral analyses, sometimes on very short notice, is greatly appreciated.

The author is indebted to the U. S. Department of Agriculture, to the Department of Entomology and to the Department of Biochemistry for the financial assistance.

The author would also like to thank his wife, Vandana, for her patience and understanding during the course of this work.

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CHAPTER I

INTRODUCTION

The greenbug, Schizaphis graminum (Rondani), is a serious pest of small grains in the central and southwestern United States. Since it was first described in Italy in 1852 and first reported from Virginia in the United States in 1882, periodic extensive outbreaks resulting in losses amounting to millions of dollars have occurred in this country. During the outbreak of 1968 a new greenbug biotype, often called the "sorghum greenbug" or "biotype C", did appreciable damage to sorghum, Sorghum bicolor (L.), a cultivar previously considered only an incidental host of greenbugs. The greenbug "biotype C" caused an estimated loss of 13.5 million bushels of grain sorghum in Kansas in 1968. In the same year about 4.5 million acres of sorghum crop were infested in Texas alone.

There are winged and wingless forms of the greenbug. All the wingless forms are females which give birth to living young. Greenbugs are pale green when newly hatched, and develop a dark green stripe on their backs when fully grown. The optimum temperature for reproduction and development is about 75°F. Reproduction of the greenbug ceases at 98°F if the host plant is wheat. On sorghum, however, these aphids survive at temperatures as high as 110°F. A single female produces about 80 offspring during a 25 day reproduction period.

Controls for the greenbug have been developed, but they are not

always dependable. The most conventional technique involves the use of insecticides such as Parathion, Disulfoton or Endrin. The use of insecticides is limited by high cost in much of the small grain growing areas of the southwest and by ineffectiveness in cold weather during which greenbugs normally develop and reproduce in fall sown grain. Because of their long range impact on the ecology and the environment, serious concern is growing about the unrestricted large scale usage of the synthetic insecticides. Because of the finite possibility of these insecticides being transferred into man, livestock and other beneficial creatures, their use is becoming unpopular.

A greenbug control procedure that offers considerable promise is plant resistance, especially in barley and sorghum. A full use of this approach can be made only after the biochemical basis of greenbug resistance in some lines of the small grains can be determined. Benzyl alcohol has been implicated as a factor in the natural resistance of barley to the greenbug biotype C. This study was undertaken to examine the metabolism of exogenously applied benzyl alcohol in barley, sorghum, and wheat. The major neutral metabolite of benzyl alcohol was fully characterized and its bioactivity against the greenbug was investigated.

CHAPTER II

REVIEW OF LITERATURE

Insect Host Plant Interactions, A General Survey

The relationship between insects and plants may be divided into two major functions; (a) host selection by the insect and (b) resistance to the insect by the plant. These two aspects are, however, not fully separable. Thus analysis of the causes of plant resistance must also include the consideration of the behavioristic and physiological characteristics of the insect. On the other hand studies of host selection must also include the role of the plant characteristics which tend to reduce the ultimate suitability of a plant to serve as a host for a given insect species.

In a very general sense plant resistance can be defined as the summation of all heritable characteristics that would reduce the probability of successful utilization of that plant (or its species) as a host to an insect species. Thus successful utilization of a host plant by an insect depends on the existence of a "proper fit" between the biological characteristics of the insect and the plant.

The role of a given plant characteristic on an insect-plant relationship is dependent upon the effects of that property on the behavioristic and developmental physiology of the insect. Hence one may consider host plant resistance being caused by a "partial fit" or "no fit" relationship between the requirements of the insect and the

correlative characteristics of the plant.

Many divergent postulates and theories have been proposed by different workers in order to rationalize the fundamental basis of host plant relations of insects, especially host specificity and resistance. As early as 1916, Comes (1) suggested that acidity might be the plant's natural defense mechanism against its enemies. The oldest view is that an insect's normal host plant satisfies fully the insect's specific nutritional and ecological requirements, whereas these requirements are not met in the complete sense by other nonhost species. According to this theory, the insect recognizes its host plant by some sort of botanical instinct. Back in 1920, Brues (2) suggested that the botanical instinct may be nothing more than extreme sensitivity to a complex of chemical and physical stimuli emanating from the plant.

It was suggested by Davidson (3) that the favorable development and reproduction of aphids on certain plants was probably associated with the nature of cell sap. The presence of chemicals in woods was considered by Fullaway (4) as a factor for the resistance of certain woods to termites. Withycombe (5) observed that susceptible sugar-cane was more attractive to the sugar-cane froghopper and suggested that the water content of the plant was involved in insect resistance. Mumford (6) suggested that the amount of reducing sugar was related to the resistance of sugar-cane to froghopper. The excess sugar was stored in the leaves of the sugar-cane in the form of starch during the day, and at night this starch was converted into reducing sugar. It was at night, when these reducing sugars were most abundant, that the froghopper fed. In 1930, Mumford (7) reported that the susceptibility of the cotton plant to attack by sap-feeding insects such as

various species of thrips was related to the water balance of the plant. He observed that plants suffering from water shortage were surely more attractive to the attacking thrips.

Fraenkel (8) postulated that host specificity and resistance were caused by the presence of "secondary" biochemicals within the plant tissues. According to this investigator, an insect's nutritional requirements do not contribute to the host plant specificity. Rather the secondary chemicals such as essential oils, alkaloids, glucosides, saponins, tannins and organic acids provide the stimuli to which the insects respond and they may use these as sensory clues in order to identify their host plants. This theory was found to be untenable because it was proven beyond doubt that feeding stimulants that play an important role in the insect's host plant comsumption are also very frequently nutritionally important components.

A "dual discrimination" theory of aphid host selection was later proposed by Kennedy (9). He postulated that in addition to specific stimulatory substances (compounds of no nutritional significance but governing botanical preferences), primary plant substances such as amino acids which serve the metabolic needs of plants as well as insects also play an important role in the aphid host interactions.

Plant Resistance to Oviposition

The first stage is the insect-plant relationship at which the plant may show resistance to oviposition for the insects that lay their eggs on or near the plants. Oviposition involves the orientation of the insect to the whole plant followed by its orientation to different plant parts in order to select a specific oviposition site. After

depositing the eggs, the insects leave the site of oviposition. Chemoreceptive and/or visual stimuli are probably involved in the initial orientation of an insect to a prospective host. Thus volatile chemicals emanating from corn foliage were demonstrated by Moore (10) to play a role in the orientation of moths of the European corn borer, Ostrinia nubilalis. Perron and coworkers (11, 12) found that the plant resistance to the onion maggot, Hylemya antiqua, was entirely due to oviposition. There was no significant difference in the nutritional values of the varieties for the maggots nor was there any evidence of antibiosis.

In addition to chemical stimuli, visual factors also influence the ovipositional behavior of an insect towards its host. Thus by passing incident light through selective filters, Horbes (13) was able to change the relative attractiveness of different varieties of wheat to the wheat stem maggot, Meromyza americana. Early orientation of corn earworm moths, Heliothis zea, to corn plants is influenced by the wave length and intensity of light reflected from the plant foliage (14). One should, however, remember that color alone does not determine host plant specificity but it simply influences early stages of orientation.

After selecting the host, the insects deposit their eggs on selected parts of the plant and not indiscriminately over various surfaces of the plant. Here again chemotactic, tactile or visual factors may be involved in the site selection and subsequent egg deposition. The corn earworm, Heliothis zea, requires a villous substrate on which to oviposit in addition to appropriate chemostimuli (15). It is found that the moth must maintain a firm tarsal

grip during egg deposition and hence needs a fibrous surface. Under field conditions, this requirement is met by the corn silks. Gupta and Thornsteinson (16) observed that the diamond-back moth, Plutella maculipennis, laid its eggs preferentially in small cavities and crevices on hosts that contained isothiocyanates. The moth could be induced to oviposit on nonhosts that had been treated with allyl isothiocyanate. These workers also made an interesting observation that allyl isothiocyanate treatment of tomato leaves did not render them susceptible to oviposition, presumably because of the presence of chemicals having a deterrent effect on egg deposition. They concluded that oviposition by the diamond-back moth is regulated by both positive and negative chemotactic factors as well as by tactile factors such as surface characteristics. Resistance to oviposition is thus an important aspect of the overall resistance of plants to insects.

Plant Resistance to Feeding

Four different steps involved in the feeding process are (a) host plant recognition and orientation, (b) initiation of feeding, (c) maintenance of feeding and (d) cessation of feeding followed by dispersal. Plant resistance may result from the plant's failing to provide the releasing stimuli required for one or more components of the feeding sequence or by the possession of characteristics having adverse effects on the feeding activities.

The feeding behavior of the larval European corn borer, Ostrinia nubilalis, has been studied very extensively. The larva is essentially polyphagous. It can feed and grow on a wide variety of plants and is known to cause severe economic damage on such diverse crops as potatoes,

peppers, chrysanthemums, dahlias, millet, sorghum and corn. The orientational phase of the borer's feeding is considered due to two very widely distributed plant biochemicals, β, γ-hexenol and α, β-hexenal. For the initial biting response, no specific incitant has yet been found. However, once feeding occurs, maintenance of feeding depends on the presence or absence of feeding stimulants or deterrents in the dietary substrate. Sugars such as glucose, fructose and sucrose act as feeding stimulants. It has been shown that corn borer larvae tend to feed most intensively on plant parts containing the highest concentration of sugars (17). Amino acids such as L-alanine, L-serine, L-threonine and L-methionene act as feeding stimulants whereas β-alanine, L-trytophan, L-phenylalanine and L-arginine act as feeding inhibitors.

Plant phospholipids (lecithins and phosphatidyl inositol) were found to be feeding stimulants for grasshoppers, Melanoplus bivatattus and Cammula pellucida (18). These plant phospholipids were isolated from wheat germ oil by chromatographic techniques. The lecithins and phosphatidyl inositol were shown to evoke striking feeding activity from older nymphs and adults for the two species of grasshoppers. This feeding activity was much more pronounced in the male nymphs than in the female nymphs.

In a study of the wireworm's response to chemical stimulation, Thorpe and Crombie (19) found that aqueous extracts of potato tubers, carrots, sugar-beet tap roots and wheat stimulated the biting of this insect. The aqueous extracts were found to contain glucose, fructose and sucrose. Other biochemicals causing biting were fats and fatty acids such as triolein, oleic acid, linolic acid and linolenic acid. Host plant specificity for the Mexican bean beetle, Epilachna

varivestris, which feeds selectively on certain species of Phaseolus genera of beans has also been investigated (20). The cyanogenetic glycosides phaseolunatin and lotaustrin, found in Phaseolus tissue, act as feeding incitants and stimulants. At high concentrations these glycosides inhibited feeding and were toxic, suggesting that bean varieties containing high levels of these glycosides might be resistant to the Mexican bean beetle.

In the case of sweet clover weevil, the feeding inhibitor has been found to be ammonium nitrate which is present only in the resistant variety of sweet clover. A number of plant alkaloids are known to exert adverse effects on larval feeding. Wada and Munakata (21) isolated a phenolic alkaloid, isoboldine, from the leaves of Cocculus trilobus which acts as a feeding inhibitor against Trimeresia miranda and Prodenia litura.

Biophysical Resistance

The physical form and tissue structure of plants undoubtedly influence their utilization as insect hosts. Unfortunately very little experimental work has been done to determine the importance of biophysical factors in host plant selection and plant resistance. The number, size and position of resin channels in pine needles play a part in resistance to the pine shoot moth and the pine needle miner. The higher rate of resin flow in resistant pine species tends to drive the larvae out of their mines by sheer physical force but it may also be an irritant (22, 23). Another example of physical resistance is found in wheat. In general the solid stemmed varieties are more resistant to the stem sawfly, Cephus cinctus, than are the hollow stemmed strains.

It appears that the sawfly eggs are mechanically damaged and dessicated more readily in solid than in hollow stems (24).

Biochemical Resistance

Plant biochemicals that have adverse effects on insect feeding behavior may thereby reduce the probability of survival due to starvation or semistarvation. Biochemicals of plant origin may also interfere with the insect's physiological processes underlying growth and reproduction. The physiological effects may be caused by metabolic inhibitors in the plant tissues, or by the plant's failing to provide specific nutrients required by the insect. Very young corn plants are highly resistant to the establishment and survival of larvae of the European corn borer. Some genetic lines of corn become highly susceptible as they mature whereas others retain much of their juvenile resistance. Beck and Stauffer (25) found three structurally related borer-toxic substances in the tissues of young corn plants and borer resistant varieties. The ether soluble fraction was found to contain two resistance factors termed RFA (6-methoxybenzoxazolinone) and RFC (2,4-dihydroxy-7-methoxy-1,6-benzoxazine-3-one), the latter being a precursor of RFA. The ether insoluble resistance factor, RFB, is the glucoside of RFC. Which of these three factors is most potent is still an unresolved controversy although all three compounds have been found to exist in vivo. Borer resistance has been found to be dependent upon the presence of an effective concentration of resistance factors in the right tissues at the right stage of growth.

Nutritional Deficiencies as a Factor in Resistance

The food requirements of insects for growth and reproduction differ greatly among different species of insects; but generally they include protein, carbohydrates, free amino acids and certain vitamins or vitamin like substances. Different varieties of a single host species may differ in quality, quantity or availability of foods for the insect. A host plant is fully adequate only if it provides the nutritional components in addition to the chemostimulants, physical factors and microenvironmental factors. Hence a resistant plant may or may not be nutritionally adequate. However in some cases the resistance may be related to the specific nutrients required by an insect.

Auclair and coworkers (26) found that pea varieties resistant to the pea aphid were deficient in amino acids. Aphids on resistant plants tend to grow more slowly than normal, secrete less honeydew and produce fewer progeny. Experiments in which pea aphids were fed on pea leaves perfused with selected amino acids (glutamine, asparagine and homoserine) gave results that supported the rationale that resistance in this case is at least partially nutritional.

Chromatographic comparison of the extracts of wheats that were susceptible or resistant to Hessian fly larvae, Phytophaga destructor, showed that the resistant variety lacked the sugar allulose and the polyhydric alcohol sorbitol (27). However the investigators were uncertain of the significance of these differences to plant resistance.

Thus host plant selection is not a simple process. Rather it involves a large number of factors, harmonious coordination of which brings about the recognition and final selection of the food plant.

Thus a plant from which a feeding inhibitor cannot be isolated may still be resistant to an insect either due to the lack of an attractant or a stimulant, or the presence of a physiological inhibitor, or a physical characteristic such as toughness or simply due to decomposition of the feeding inhibitor because of a poor isolation procedure.

Greenbug Related Work

Several workers have investigated various aspects of the biochemical and physiological interactions between the greenbug aphid and the small grains. The reaction of small grains and grain sorghum to three greenbug biotypes was reported by Wood and coworkers (28). Hackerott and his associates reported (29) that greenbug resistance in sorghum appears to be conferred by dominant genes at more than one locus. The effectiveness of resistance to greenbugs (biotype B) in wheat, barley, rye and sudangrass was compared with resistance to greenbugs (biotype C) attacking sorghum (30). Ward and coworkers (31) investigated the use of several insecticides to control the greenbug infestation of sorghum. Physiological changes in barley induced by greenbug feeding stress were studied by Gerloff and Ortman (32). These researchers observed that the greenbug feeding stress on the first foliar leaf of barley caused a severe deterimental decline in chlorophyll content and in the rate of photosynthesis.

Todd and coworkers (33) studied the toxicity of various phenolic and flavonoid compounds to the greenbug aphid (biotype B). Based on the results of the feeding studies, these investigators suggested that the resistance of some barley varieties could partially be due to the presence of some phenolic and flavonoid substances in quantities

sufficient to retard the insect's growth and reproduction. A detailed study of the resistance in barley to the greenbug (biotype C) was done by Chan (34). No specific biochemical substance that could account for the plant resistance was, however, found. In 1972, Juneja and coworkers (35) reported that benzyl alcohol might be one of the biochemical factors responsible for the resistance to the greenbug observed in some varieties of barley. In addition to reducing the rate of greenbug reproduction, benzyl alcohol was found to protect the treated plants against the greenbug attack.

CHAPTER III

METHODS AND MATERIALS

Materials

Isogenic greenbug resistant and susceptible (Iso(R) and Iso (S)) barley varieties were developed by the Agronomy Department of Oklahoma State University (35). Susceptible wheat (Triumph) and sorghum (Wheatland) were also used for this study. The plants were grown from seed in a growth chamber under controlled conditions; humidity 40%, day temp. 85°F, night temp. 65°F, 16 hour light period. Seedlings 8-10 days old and 5-6 inches tall were generally employed. (^{14}C-carbinol)-benzyl alcohol was purchased from Radiochemical Center, Amersham. α And β glycosidases were obtained from Sigma Chemical Company. All other chemicals used were of reagent grade.

General Methods

Aqueous solutions were concentrated at or below 42° on a rotary evaporator. Acid hydrolysis was normally accomplished by heating the substrate with 1N HCl for 45 min in a boiling water bath (36).

Descending paper chromatography was done on Whatman No. 1 Chromatographic paper. Glycosides and sugars were detected with $AgNO_3$/NaOH reagent, according to the procedure of Trevelyan et al., (37).

Mass spectra were determined with the prototype (38) of the LKB-9000 gas chromatograph-mass spectrometer with the use of the direct

inlet probe. Infrared spectra were determined with a Perkin-Elmer 457 grating infrared spectrometer in KBr pellets prepared with a microsampling kit. NMR spectra were obtained on a Varian-100 NMR spectrometer in acetone-d6. Elemental analyses were performed by Galbraith Labs, Inc. Radioactivity measurements were made in a toluene-ethanol cocktail (60%-40% v/v) containing 4 g PPO and 200 mg POPOP per liter, using a Packard Tri-Carb Liquid Scintillation Spectrometer. Analytical TLC was performed on silica gel coated Eastman Chromatogram sheets No. 6060. Quantagram's precoated glass plates PQ1 were employed for preparative TLC. Gas liquid chromatography of the aglycone was performed on a Barber-Coleman instrument using a 6' x ¼" glass column packed with 10% carbowax - 20 M on gas chrom-Q. Borate ion-exchange chromatography was employed for the purification of the neutral metabolite and identification of its monosaccharide constituent in accordance with the procedure described by Lee *et al.* (39) using a Technicon Model SC-1 autoanalyzer.

Periodate oxidation was performed with sodium meta-periodate for a period of 24 hrs at room temperature in the dark. Utilization of periodate was followed by the method of Avigad (40). Formaldehyde was determined with chromotropic acid by the method of Frisell *et al.* (41). Formic acid produced during the periodate oxidation was determined by addition of 1 ml of ethylene glycol to 5.0 ml aliquots of the reaction mixture and after 30 min titrating the sample with 0.001N NaOH to pH 8.0.

Administration of the Labelled Compound

For analytical experiments, 4 microliters of an aqueous solution containing 1% benzyl alcohol was injected into the stem of each seedling. After treatment the plants were allowed to continue growing for a

predetermined time. A different procedure was used for experiments designed for the isolation of the neutral metabolite. After removing the plants from soil, the roots were thoroughly washed in luke-warm water. The roots were blotted dry with paper towels and the plants transferred into small glass bottles (2" in diameter and 3" in height). The solution of benzyl alcohol (1%) containing some ^{14}C tracer was slowly pippeted onto the roots (generally 1 ml of solution per 20-25 seedlings was used). The bottles were wrapped in aluminum foil and the plants allowed to stay in the laboratory until nearly all of the solution was taken up into the roots (3-4 hours). The roots and the lower portion of the stem were then covered with a nutrient solution and the plants were transferred to the growth chamber where they were kept until harvest (48 hrs after treatment).

Work Up Procedure for Analytical Experiments

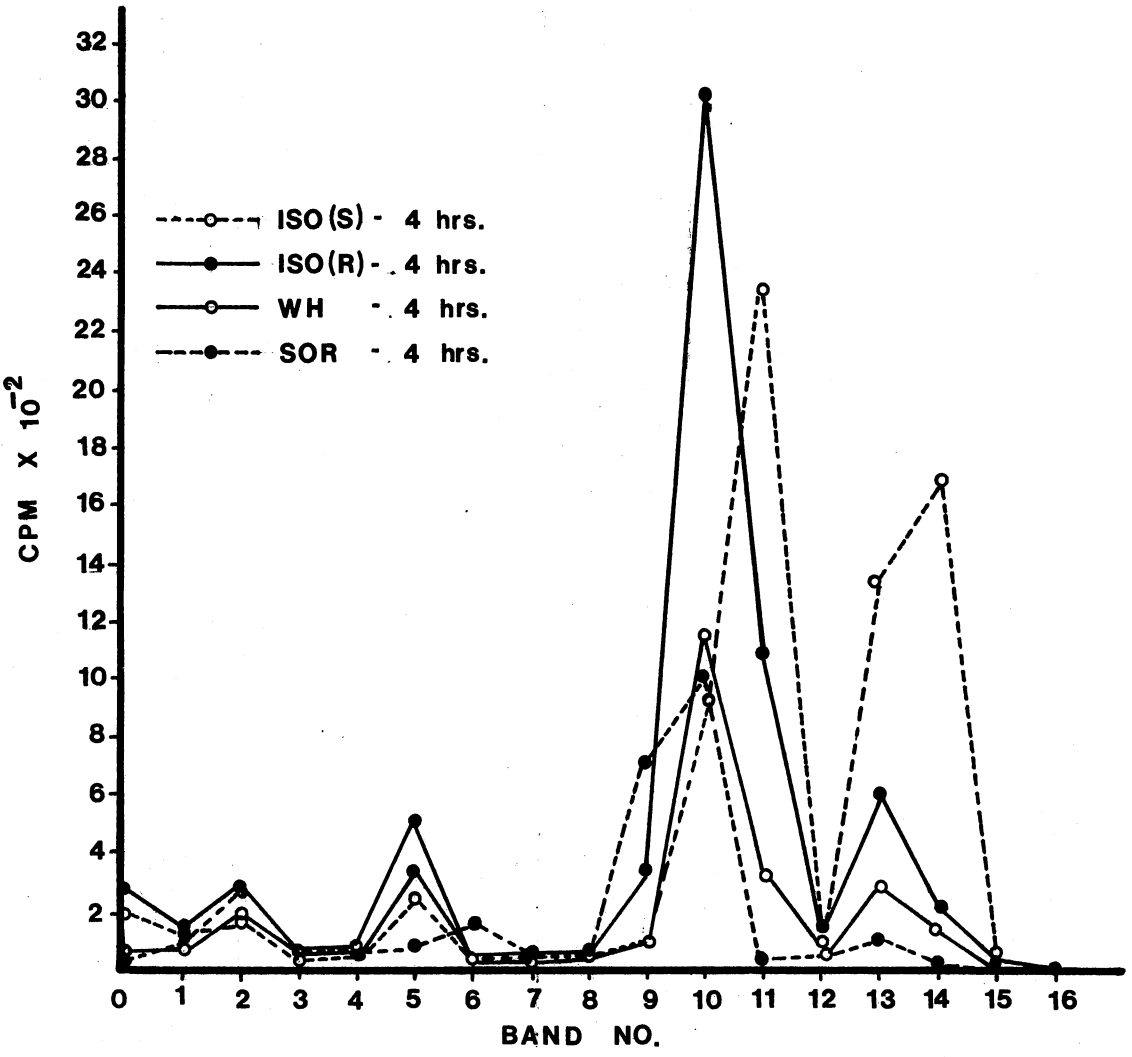
The stems and leaves were frozen with liquid nitrogen in a mortar and pulverized with a pestle. The plant material was then stirred with water and centrifuged in a clinical centrifuge (at 2-3°C). After removing the supernatant, the residue was retreated with water and again centrifuged. The two supernatants were mixed and centrifuged at 20,000 g (20 min). The clear supernatant was removed and evaporated to dryness on a rotary evaporator (bath at 42°C). The residue was extracted with 1 ml of methanol in two portions, the methanol extract being separated each time from the insoluble material by centrifugation. An aliquot of the MeOH extract was either spotted or streaked on the origin line of a 2.5 x 20 cm section of a 20 x 20 cm TLC plate. Eight samples were analyzed per plate. The plate was then developed (ascending) in

methanol:toluene (2:3) until the solvent had traveled 16 cm above the origin. After drying, the plate was cut into eight 2.5 x 20 cm strips. Each strip was then divided into seventeen pieces, 2.5 x 1 cm each, the first piece (0.5 cm on either side of the origin line) being numbered 0 and the last one (0.5 cm on either side of the front) being 16. Each of the pieces was placed in a scintillation vial and treated with 1 ml of MeOH. After adding 10 mls of the standard cocktail, the radioactivity was counted and cpm were plotted against band number. One such profile corresponding to 4 hrs metabolism of benzyl alcohol in barley, wheat and sorghum is shown in Fig. I. Four peaks marked I, II, III, and IV were produced by the radioactivity in bands 0-1-2, 5-6, 8-9-10, and 13-14. Relative distribution (Tables I and II) was calculated by dividing the radioactivity under each peak by the sum of radioactivities under all four peaks.

Isolation of the Neutral Metabolite

48 Hrs. after treatment, the plants were removed from their containers, and the nutrient solution retained on the roots was removed by washing. The leaves and stems were ground with liquid nitrogen to a fine powder. Water was added (5 ml/g fresh weight) and the slurry was stirred at 2-3°C for 1-2 hrs. The brei was evaporated at 42°C on a rotary evaporator. The plant residue was again mixed thoroughly with water and filtered through 8 layers of cheesecloth. This treatment was repeated. The total filtrate upon evaporation gave a yellow viscous residue that was treated with a limited amount of water and centrifuged (20,000 xg). The dark yellow supernatant was evaporated, and the residue thus obtained was stirred with methanol. The white insoluble

Figure 1. Comparison of Metabolites Formed from (^{14}C)-Benzyl Alcohol 4 hrs after Injection in Wheat, Sorghum and Greenbug-Resistant and Susceptible Barley. Seedlings (8-10 days) of all four varieties were injected with 4 µl of 1% (^{14}C-carbinol)-benzyl alcohol (700 µc/ml). Seedlings were harvested 4 hrs after injection and methanol extracts were subjected to ascending TLC in methanol-toluene (2:3); the TLC plate was cut into strips which were counted by liquid scintillation. (See Methods for details). Two seedlings were combined for each point. Greenbug-susceptible (Iso (S)) barley ---0---, greenbug-resistant (Iso(R)) barley ——●——, wheat ——0——, sorghum ---●---.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ISO(R) - 4 hrs.
WH - 4 hrs.
SOR - 4 hrs.
CPM X 10^{-2}
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0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32

TABLE I

COMPARATIVE METABOLISM OF ^{14}C-BENZYL ALCOHOL IN WHEAT, SORGHUM AND GREENBUG-RESISTANT AND SUSCEPTIBLE BARLEY

Plant[1]	Time After Injection Hrs.	Percent of Recovered ^{14}C in Metabolite			
		I	II	III	IV
WH	4	12.4	20.4	52.8	14.4
SoR	4	17.0	11.3	67.1	4.6
R	4	11.7	6.9	69.1	12.3
S	4	6.9	4.5	47.0	41.6
WH	24	57.1	9.1	29.0	4.8
SoR	24	43.4	11.3	38.2	7.1
R	24	42.5	5.9	49.4	2.2
S	24	42.9	4.0	51.3	1.7
WH	48	81.0	7.5	10.0	1.4
SoR	48	42.1	25.7	31.0	1.2
R	48	63.9	6.5	28.8	0.8
S	48	59.4	6.2	33.2	1.2
WH	72	82.3	10.2	5.8	1.7
SoR	72	44.4	27.5	27.9	0.2
R	72	70.2	6.2	23.3	0.2
S	72	67.0	6.1	26.2	0.7
WH	96	86.4	7.7	4.9	1.0
SoR	96	50.5	24.7	24.2	0.5
R	96	75.5	7.3	16.9	0.3
S	96	72.9	6.5	20.3	0.2

[1]WH-Wheat, SoR-Sorghum, R-Isogenic resistant barley, S-Isogenic susceptible barley.

TABLE II

COMPARATIVE METABOLISM OF ^{14}C-BENZYL ALCOHOL IN WHEAT AND GREENBUG-RESISTANT BARLEY

Plant[1]	Time After Injection Hrs.	Percent of Recovered ^{14}C in Metabolite			
		I	II	III	IV
WH	6.5	21.5	21.5	48.7	8.7
R	6.5	16.6	9.4	69.2	4.9
WH	96.0	86.8	7.3	4.7	1.2
R	96.0	76.6	6.6	15.9	0.9

[1]WH-Wheat, R-Isogenic resistant barley.

material was removed by filtration through a sintered glass funnel. The residue left upon removal of solvent from the filtrate was dissolved in a minimum amount of water and loaded on a Bio-Rad AG-50-W x 8 column (H^+ form, 200-400 mesh, 35 x 1.5 cm) which was eluted with water. The effluent from the cation exchange column was concentrated to a small volume and fractionated on a Bio-Rad AG-1 x 8 column (formate form, 200-400 mesh, 35 x 1.5 cm). The neutral metabolite was eluted from the anion exchange resin with water. The product thus obtained was further purified by preparative TLC coupled with autoradiography, followed by borate ion-exchange chromatography. However, by employing relatively large columns (55 x 3.1 cm for the cation exchange resin and 54 x 1.5 cm for the anion exchange resin) we were able to isolate a neutral metabolite that did not require further purification.

Synthesis of Benzyl Glucoside

Emulsion (almond) catalyzed synthesis of β-benzyl-D-glucopyranoside has been reported by Bourquelot and Brickel (42) as well as by Slotta and Heller (43). In both methods several weeks were required to produce the glucoside in low yields. Synthetic attempts using Dowex-50 (H^+) as the catalyst (44) did produce β-benzyl-D-glucopyranoside but in poor yield. Finally the procedure of Purves and Hudson (45) for the preparation of β-benzyl-D-fructopyranoside was modified in order to prepare our compound. Benzyl alcohol (100 mls) containing 0.2 to 0.3 N HCl (dry gas) was added to powdered anhydrous dextrose (5 g) in a 500 ml glass bottle. The bottle was stoppered with a silicone rubber stopper and shaken vigorously on a reciprocal mechanical shaker for 16 hrs. The solution was filtered on a Buchner funnel and the undissolved glucose

washed with three portions of benzyl alcohol (10 ml each). Approximately 1.5 g of glucose was thus recovered. The filtrate was treated with 10% $NaHCO_3$ solution (80 mls). The mixture was then evaporated until no more water distilled over. The precipitated $NaHCO_3$ and NaCl were removed by filtration and thoroughly washed with fresh benzyl alcohol (20 mls). The total filtrate (~150 mls) was diluted with four volumes of benzene and extracted thrice with water (total 200 mls). The aqueous solution was evaporated under reduced pressure to give an oily residue that was washed with ether (15 mls). After removing the ether, the viscous material was dissolved in a small amount of water and freeze-dried. The glassy product thus obtained was crystallized from ethyl acetate-ether and dried under vacuum. Yield = 2.3 g. The product though free of glucose was a mixture of α and β anomers in nearly 1:1 ratio.

Enzymatic Hydrolysis of Synthetic Mixture and Plant Metabolite

The metabolite isolated from the plant (5.0 mg) and the synthetic mixture of α and β benzyl-D-glucosides (10.0 mg) were each dissolved in acetate buffer, pH 5.0 (1 ml). 20 µl of β-glucosidase in H_2O (5 mg/ml) and 5 µl of toluene were added to each substrate solution. They were then incubated at 37° for 24 hrs. Corresponding controls were run in a similar manner except that the enzyme was omitted. The aglucone was extracted twice with ether (2 x 1 ml) and analyzed by GLC. After removing residual ether in a stream of N_2 gas, the aqueous solution was loaded on a small column (7 mm x 55 mm) the lower half of which was packed with Ag-1 x 8 (formate, 200-400 mesh) and the upper half with

AG-50-W x 8 (H^+, 200 mesh). The column was then eluted with water (20 ml). The solid obtained on evaporation of the effluent was dissolved in 1.5 ml of 50% ethanol. An aliquot (15 µl) of this solution was analyzed on a borate ion exchange column coupled to a Technicon carbohydrate analyzer.

CHAPTER IV

RESULTS AND DISCUSSION

Time Course of Benzyl Alcohol Metabolism in Barley, Wheat and Sorghum

In the analytical experiments an aqueous solution of benzyl alcohol (1%) containing {^{14}C}-benzyl alcohol (~6.5 µc/mmole) was injected with a micro syringe directly into the stems of 8-10 day old seedlings. The plants were harvested 4 hrs, 24 hrs, 48 hrs, 72 hrs and 96 hrs after injection.

The methanol extracts (see Methods) containing various metabolites of benzyl alcohol were examined by TLC. The radioactive metabolites were located by liquid scintillation counting of 1 cm wide strips of the TLC plates. Plots of radioactivity vs R_f revealed four different compounds or groups of compounds with R_f values of 0.0-0.16 (I), 0.28-0.41 (II), 0.59-0.72 (III) and 0.78-0.91 (IV). A plot of the radioactivity in I, II, III and IV 4 hrs after injection of benzyl alcohol -^{14}C into resistant and susceptible barley, and wheat and sorghum is shown in Figure I. The relative distributions of these components in all four plant varieties at periods of 4 hrs to 96 hrs following injection are summarized in Table 1.

The compound(s) in peak III is neutral since it was not retained on either cation or anion exchange resin and could be eluted from either of these columns with deionized water. The compound(s) of peak I are

all acidic since they were bound on AG-1 x 8 (formate), from which they could be eluted with 2 to 4 N formic acid or 4 N ammonium formate. From relative mobilities it may be assumed that metabolite IV is also neutral whereas metabolite II is probably acidic in nature. The drop in the level of IV in all plants within 24 hrs to insignificant amounts suggests that this compound(s) is a transitary intermediate in the metabolism of benzyl alcohol. The amount of metabolite II varies only slightly in wheat and barley, whereas, in sorghum it appears to build up with time. The time course metabolism of benzyl alcohol reveals that the largest changes occur in the relative distribution of metabolites I and III. Although initially III is produced in relatively large amounts, it's concentration rapidly decreases with time, with a corresponding increase in the level of I. These time dependent changes in III and I probably indicate their product-precursor relationship. The most striking difference between plant varieties observed in these experiments was that III decreased in wheat at a faster rate than it did in sorghum or barley. Thus in wheat, III is reduced from 53% (at 4 hrs) to 5% (at 96 hrs). Whereas in the same time interval it drops in sorghum and barley from nearly 70% to about 20%. The net result of this change is that after 48 hrs sorghum and barley have 3 to 4 times more radioactivity in III than does wheat. In these experiments, two seedlings of each species were used for each timed run. To provide a better statistical view, wheat and Iso(R) barley were again compared for patterns of metabolites, using five seedlings per run. Only two time periods, 6.5 hrs and 96 hrs were investigated, the results are summarized in Table II. Results that coroborated the data shown in Table I were obtained, the decrease of III in wheat being relatively much greater than that observed

in barley.

Another important result of this experiment is the finding of little or no qualitative or quantitative differences in the metabolism of ^{14}C-benzyl alcohol in isogenic resistant and susceptible barley plants at 24 or more hrs after injection (Table I). This finding, together with the previous observation of the presence of free benzyl alcohol in resistant but not in susceptible plants (1), strongly suggests that the gene which confers greenbug resistance to barley is concerned with the synthesis rather than the subsequent metabolism of benzyl alcohol.

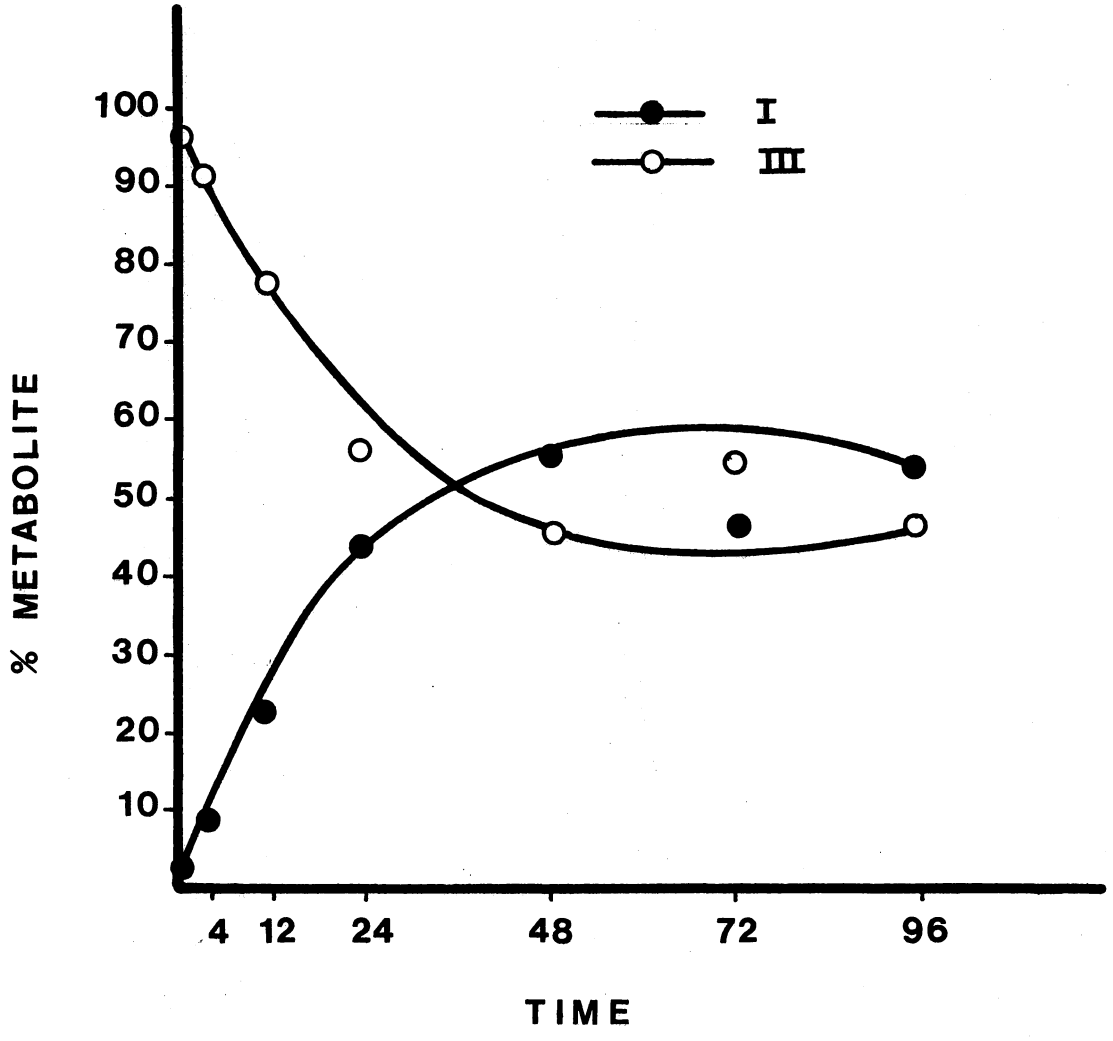
Conversion of Compound III to Compound I

As mentioned above a precursor-product relationship appears to exist between III and I. This was clearly demonstrated when III, isolated in crude form from barley (both Iso (R) and Iso (S)) and partially purified by ion exchange chromatography, was reinjected into Iso(R) plants. Formation of I together with the corresponding dissappearance of III is shown in Fig. 2. This suggests that III is converted into I until some form of equilibrium is established.

Isolation and Structure Determination of Neutral Metabolite

Since metabolite III is the major product formed in the early stages of benzyl alcohol metabolism, its purification and identification were undertaken. In order to isolate this metabolite, preparative scale experiments in which a 1% aqueous solution of benzyl alcohol was fed to the roots of Iso(R) plants were carried out. Partial purification of the neutral metabolite was achieved on ion exchange columns (see

Figure 2. Conversion of Neutral Metabolite (III) into Acidic Metabolite(s) (I) in Greenbug-Resistant Barley. Carbon-14 labeled metabolite III (now identified as β-benzyl-D-glucoside) was isolated from barley seedlings 4 hrs after injection of 10 seedlings with 4 µl of 2% (^{14}C)-benzyl alcohol, and purified by cation and ion exchange column chromatography (see Experimental "Isolation of Neutral Metabolite"). Resistant barley seedlings were injected with 4 µl of a solution of (^{14}C)-metabolite III containing 1.2 x 10^6 cpm/ml. Three seedlings were harvested at each time period indicated and analyzed for metabolite I and III by thin layer chromatography (see Experimental "Work Up Procedure for Analytical Experiments").


100
90
80
70
60
50
40
30
20
10
% METABOLITE
I
III
4
12
24
48
72
96
TIME

Methods). Further purification was obtained by preparative TLC coupled with autoradiography. The product thus obtained gave a single round UV absorbing spot when examined by TLC in three different solvent systems, A-ethyl acetate:cyclohexane (4:1), B-ethyl acetate:MeOH:H_2O (16:1:1), and C-ethyl acetate:pyridine:H_2O:MeOH (5:1:4:3), the R_f's being 0.13, 0.55 and 0.93 respectively. Two dimensional TLC using solvent system D-ethyl acetate:cyclohexane:MeOH (4:2:2) also gave a single spot under UV light. It contained 94% of the radioactivity initially spotted, the remaining 6% being in the tailing portion of the spot. The purified metabolite gave a positive anthrone test. Fehling's test for the presence of reducing sugar was positive only after acid catalyzed hydrolysis of the metabolite. Specific color reactions (46, 47) showed the presence of an aldosugar and the absence of a pentose or a keto sugar. Based on this information, the neutral metabolite was tentatively assumed to be a benzylglucoside. The IR spectrum (KBr) contained three absorption bands of medium intensity at 13.3, 13.59 and 14.34 μ suggestive of a monosubstituted benzene ring. The two most intense ions in the low resolution mass spectrum were observed at m/e 91 and 92 which could be ascribed to benzyl and tropylium ions respectively. The fragment of highest mass had m/e of 252. This leads to a mass of 162 ($C_6H_{12}O_6$) for the sugar moiety. As it is fairly easy for a sugar molecule to lose a molecule of water during mass spectral analysis (by direct probe) a mass of 180 ($C_6H_{12}O_6$) for the glycone could also be visualized. Descending paper chromatography in solvent E-ethyl acetate:H_2O (5:3:2) and F-ethyl acetate:acetic acid:pyridine:water (5:1:5:3) again showed the neutral metabolite as a single spot. The metabolite was hydrolyzed with HCl. The aglycone was extracted into ether and was shown to be benzyl

alcohol by GLC. Paper chromatographic analysis of the hydrolysis mixture surprisingly showed two spots, the major (90%) being glucose (R_f in E and F systems were 0.29 and 0.55 respectively). The minor spot had a higher mobility (R_f = 0.46 and 0.84 respectively in E and F) than glucose.

The metabolite was resolved into two components on a borate ion exchange column attached to a Technicon carbohydrate analyzer. Only the major peak contained radioactivity. The fractions under the major component were pooled and the solution passed through cation and anion exchange resins in order to remove the buffer salts. After removing the residual boric acid as methyl borate, the residue obtained was hydrolyzed with acid. Glucose was the only sugar present in the hydrolysate as determined by paper chromatography in solvent F and by borate ion-exchange chromatography; hence, the neutral metabolite is benzyl glucoside. Obviously the neutral metabolite purified by preparative TLC retained a contaminant (probably a glycoside) that had the same mobility as the benzyl-glucoside under the conditions used for TLC and paper chromatography. However, by using ion exchange columns of larger size, benzyl glucoside of very high purity was obtained. The material thus obtained need not be further purified by preparative TLC or by other means. On hydrolysis it gives a single spot due to glucose. Only one peak is obtained when analyzed on a borate ion exchange column. Elemental analysis of this preparation after recrystallization from ethyl acetate-ether gave C=57.88% and H=6.56%. (Calculated, C=57.78%, H=6.67%)

Stereochemical Conformation at the Anomeric Carbon

Rathbone *et al.* (48) have reported NMR data on a number of deriva-

tives of α and β-D-galactopyranoses. These authors found that the anomeric proton (H-1) in α-galactopyranosides and β-galactopyranosides was observed at τ 4.75-5.2 and 5.4-5.7 respectively. The corresponding $J_{1,2}$ values were 3-4 cps and 7-8 cps respectively. NMR spectrum of the neutral metabolite in acetone-d_6 gave a doublet (1 H) at τ 5.6, $J_{1,2}$ = 7.6 cps which indicates that this product is β-benzyl-D-glucopyranoside. Acid catalyzed synthesis of benzylglucoside from benzyl alcohol and D-glucose gave a mixture of α and β anomers in nearly 1:1 ratio. When analyzed on a borate ion exchange column, the second peak, (Fig. 3) matched the plant metabolite. The plant product as well as the second peak of the synthetic mixture were completely hydrolyzed by commercial β-D-glucosidase, liberating in each case a corresponding amount of glucose. (Fig. 3) These results confirmed the β-configuration of the glucoside linkage.

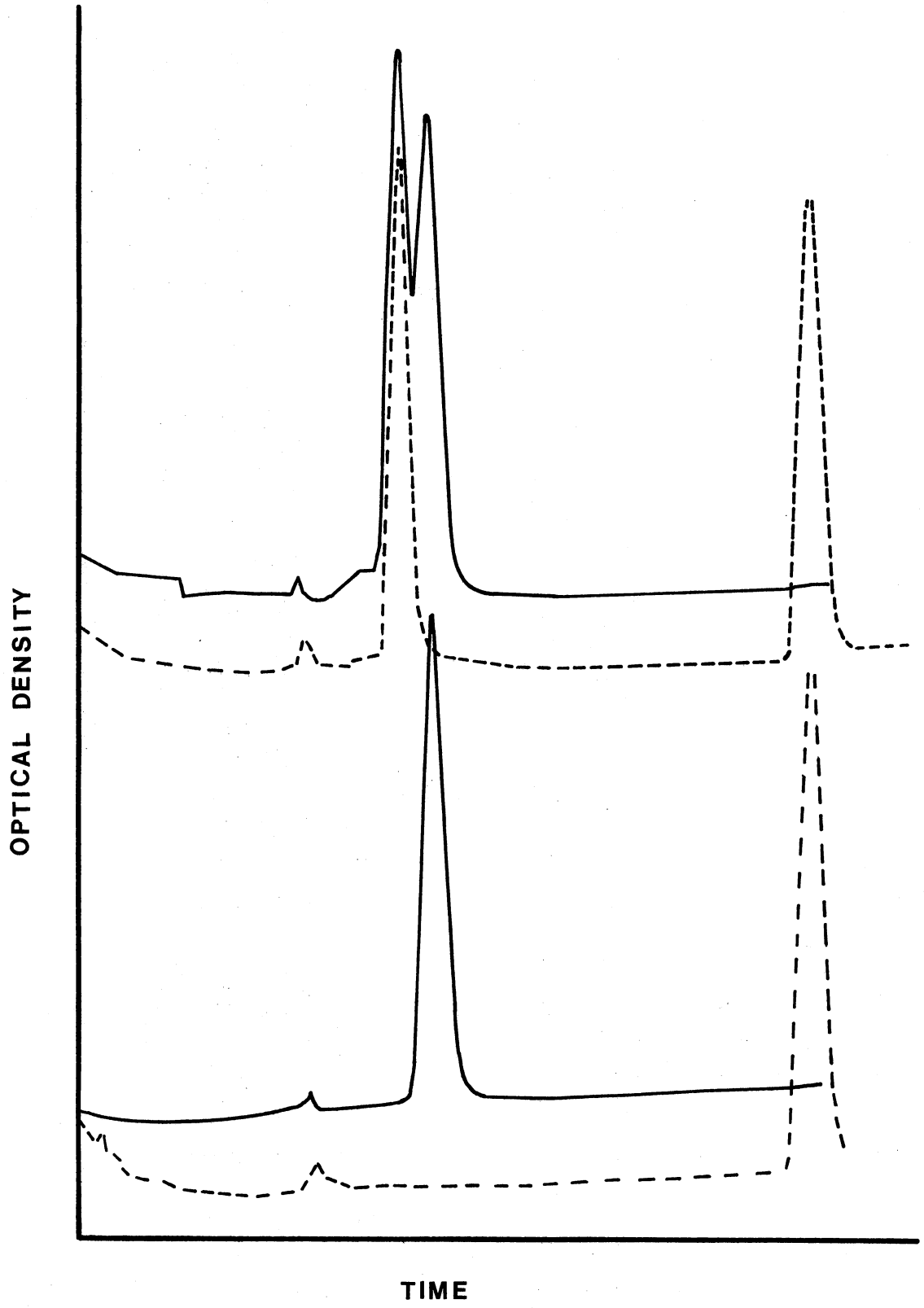
Ring Form of the Sugar in Benzyl Glucoside

The β-benzylglucoside formed in barley was identified as the pyranose form by quantitative periodate oxidation followed by formaldehyde and formic acid determination. The glucoside consumed 1.9 molar equivalents of periodate and yielded 0.92 molar equivalents of formic acid. No formaldehyde was detected. These results establish the compound as β-benzylglucopyranoside.

Biological Activity of Benzyl Glucoside

Metabolism of benzyl alcohol in animals such as rabbits and rats has been investigated by several workers (49, 50, 51). These studies have shown that a major portion of benzyl alcohol given to the animal is

Figure 3. Analysis of Synthetic and Isolated Benzyl Glucosides by Borate Ion-Exchange Chromatography before and after Hydrolysis with β-Glucosidase. Samples of synthetic benzyl glucoside (upper curves) and benzyl glucoside isolated from resistant barley after treatment with benzyl alcohol (lower curves) were chromatographed on a Technicon Model SC-1 Carbohydrate Analyzer. Solid lines show analyzer tracings before enzymatic hydrolysis. Dotted lines show analyzer tracings of samples subjected to hydrolysis by β-glucosidase. (See Experimental for details).


OPTICAL DENSITY
TIME

excreted as it's conjugate with glycine, hippuric acid. Microorganisms such as Pseudemonas and Arthrobacter are known (52, 53, 54) to oxidize benzyl alcohol to benzoic acid followed by ring hydroxylation of the latter. Very little information on the metabolism of benzyl alcohol in plants is available. Ciamician and Ravenna (55) reported that an acetone extract of maize plants which had been inoculated with a mixture of benzyl alcohol and glucose contained bound benzyl alcohol which was assumed to be present in the form of a glucoside which, however, could not be isolated in a crystalline form. The H_2O extract of the same plants was found to contain neither free nor bound benzyl alcohol. This report is surprising since β-benzyl-D-glucoside is extremely soluble in water. Germinating corn and kidney beans were found to convert benzyl alcohol into a compound that was considered to be glucosidic in nature (56).

Glycosides have been reported to have several major functions in plants (57). Glycosides have been implicated in host plant-insect interactions (58, 59). Therefore the biological activity of β-benzyl-D-glucoside on greenbugs was investigated. The effect of β-benzyl-D-glucoside on the rate of greenbug reproduction is shown in Table III. The average number of nymphs per plant at the end of 3 days of treatment was nearly the same for the treated plants and the control (H_2O). The glycoside at 1000 ppm levels was found to be phytotoxic. β-Benzyl-D-glucoside, unlike free benzyl alcohol has almost no activity in the greenbug test system (1).

TABLE III

BIOASSAY OF β-BENZYL GLUCOSIDE FOR ANTIBIOSIS AGAINST GREENBUGS

Treatment	Avg. no. progeny/plant on days after infesting			Total Progeny/plant
	1	2	3	
Benzyl alcohol at 100 ppm	2.0	3.2	3.7	8.9[a]
Benzyl glucoside at 10 ppm	3.5	3.7	5.2	12.4
Benzyl glucoside at 100 ppm	4.3	3.9	5.5	13.7
Benzyl glucoside at 1000 ppm	3.1	3.0	3.4	9.5[b]
Water only	3.3	5.3	5.2	13.8

Isogenic greenbug susceptible barley seedlings (2 weeks after planting) were individually placed on 5 x 2.5 cm glass vials with the roots submerged in the test liquid. Three adult Biotype C greenbugs were placed on each plant and the vials maintained in a growth chamber at 22°C except during progeny counts. Sixteen replications (plants) were used in each test. The β-benzyl glucoside used in this test was isolated from benzyl alcohol treated isogenic greenbug resistant barley and purified by cation and ion exchange chromatography.

[a]This value is significantly different from the water control at $P = 0.05$ by Ducans multiple range test.

[b]Benzyl glucoside was phytotoxic at this concentration.

CHAPTER V

CONCLUSION

Strains of barley which are resistant to attack by the greenbug aphid, Schizaphis graminum L., have been found to contain benzyl alcohol while susceptible strains do not (1). Under laboratory conditions benzyl alcohol lowered the reproduction rate of greenbugs on susceptible barley (1) and protected susceptible sorghum from greenbug attack under field conditions (2). These findings led to the tentative conclusion that benzyl alcohol may be responsible for the natural resistance to greenbugs present in some strains of barley and sorghum. Both resistant and susceptible strains of barley as well as wheat and sorghum metabolize exogenous (^{14}C)-benzyl alcohol into several metabolic products. In 48 to 72 hrs after treatment only traces of free (^{14}C)-benzyl alcohol can be recovered from treated plants.

The major neutral metabolite was isolated and characterized as benzyl β-D-glucoside. This metabolite did not exhibit any biological activity against the greenbug aphid.

It is possible that some of the other metabolites of benzyl alcohol may have antigreenbug activity. However, the virtually identical patterns of benzyl alcohol metabolism observed in isogenic resistant and susceptible barley, together with the previous observation of free benzyl alcohol in tissues of resistant but not of susceptible plants, are consistant with the genetic defect in susceptible strains being

located in the pathway of benzyl alcohol synthesis and with free benzyl alcohol being the plant component responsible for greenbug resistance.

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